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Isolation of *Ascochyta lentis* fungus, responsible for blight disease in lentil crop and screening of suitable fungicides for management

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Lentil is an important legume crop that is a safe source of high-quality dietary protein. Mostly fungal diseases are considered an important constraint of Lentil. Blight disease is the major issue of this important legume crop caused by *Ascochyta lentis* is an important disease caused by *A. lentis*. Samples from diseased plants were collected, and the pathogen was isolated on artificial media. After purification, the pathogen was indentured based on the growth pattern, colony shape, and spore structure. There is very fewer germplasm of Lentil available in the country. The current study shows 12 lentil genotypes (11504,12512,12514, M-09501, Pb-m-2009, M-93,12505,12503,11507,11509,10503, M-85) were screened against *A. lentis* by following RCBD under natural and artificial disease conditions. Agronomic, morphological and physiological data were recorded from field trials. Fungicides were diluted into different concentrations and were used to inhibit the growth of *A. lentis* under in-vitro conditions. Similarly, BCAs were also screened against A lentis on artificial media plates and were utilized in field conditions to manage the disease.

Keywords: *Ascochyta lentis*, Biocontrol agents, Disease resistance, and Lentil blight .

INTRODUCTION

Lentil (*Lens culinaris* L.) is considered as the oldest legume crop cultivated in South-West Asiain countries and is witnessed 7000 year old in literature. (Ahad and Matiur, 1993). Lentil is widespread through the world but mainly grown in Asian countries. World-wide, its production area is 4600k hectares which gives 4200k tonnes of lentil (FAO, 2016). Pakistan is the major lentil producer in south Asia. It is grown on 33,909 hec and 17909 tons of production. (Agriculture Statistics of Pakistan, 2019).

Lentil has many health benefits. As it belongs to legumes family, it has high value of protein content which is about 30% calories content. Besides proteins it has high amount of dietery fibers, vitanims (i.e. iron and zinc), minerals and carbohydrates. (Callaway, 2004). Considering the percentage of each nutrisious content, protein 28.6%, crude fiber 4.6%, starch is 444.3%, amaylose 36.1%, ash 3.1% and 63.1% carbohydrates, which gives 420 calories in 100 gram of cooked lentil. (Yadav *et al.*, 2017). Furthermore, lentil has low anti-nutritional factor like hemagglutinin, oligosaccharide, and flavone than most other leguminious crops. Lentil comprise of tannin in the seed coat but not in cotyledon, and their consumption is safe as they are used in the human diet after the removal of the seed coat (Srivastava and Vasishtha, 2012). Lentils have two types: large-seeded Lentils called *macrosperma* and small-seeded *microsperma*. The macrosperma compirise of tannin, that can cause digestion related problems. But on the other hand higher amount of protein, low anti-nutritious values and easily cooking make it fit for consumption.

Ascochyta blight disease of lentil was 1st reported in 1938 (Bondartzeva-Monteverde and Vassilievsky, 1940). Cultivation of lentil is reported in many areas in the world which are famous for the ciultivation of legume crops (Vandenberg, 2009). Many researchers worked on blight disease and paticularly Gossen *et al*. (1986) suggested that *Ascochyta lentis* should be synonymized with *A. fabae*.

Ascochyta blight (AB) of lentil infected by *A. lentis*, it is widespread in most lentil cultivated areas of the world which causes production losses ranging up to 70% in Canada, whereas such losses are comparoitevely less in USA, 30 to 50% while in Australia losses due to this important fungal disease reported upto 50% (Brouwer *et al*., 1995). Morrall and

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Sheppard (1981) termed isolated *A. lentis* and reported which generally, *A lentis* conidia consist od two cells with a size ranging 10-20 µm and on the other dimention upto 4-8 µm (Mean: 15.8 × 5.7 µm). This shows single spetation and rarely multi-septate conidia can exist amongst the majority of the normal two-celled condidial form. The taxonomy of *A. lentis* has been a topic of discussion during 1980 when it was considered as *forma specialis* of *A. fabae* due to high microscopic and physiological similarities (Gossen and Morrall, 1984).

In 1993, *Didymella lentis*, which is teleomorph stage of *A. lentis*, was isolated from overwintered lentil plant debries in eastern Washington. Kaiser and Hellier (1993) distinguished the teleomorph stage of *A. fabae* and *A. lentis* on the basis of pathogenicity and morphologically. Their pycnidia discharge conidia that can move short distance area with the help of rain water and wind.

A. lentis is hetero-thallic with typically two mating types (MAT1-1 and MAT1-2) (Falahati et al., 2010). Conidia can cause infection in lentil plants, it germinate at the optimum temperature of 20-25°C and in the existence of relatively high moisture in the air. *A. lentis* conidia can survive either inside the seed of crops or leftover crop debris. The disease brutally affect seed quality and yield (Iqbal and Mukhtar, 2014). Blight disease is an important economic concern in Australia, Canada, India and Pakistan (Ilyas *et al*., 2010). Gossen and Morrall (1983) reported that severe foliar infection resulted in yield losses of over 40%. Similarly, Hussain *et al.* (2016) found that 30-40% of crop losses in Pakistan due to ascohyta blight. Mostly, seed contamination is also a severe cause of losing seed quality and become less attractive in the market.

In the present study, isolation and identification of the fungal pathogen associated with lentil blight were performed

Chemicals were only sorted out in the *in-vitro* as fungicide application on spores to find the suitable dose. While BCAs were tested in dual plates assays to find the potential Biocontrol agent and, later, were applied in the field to manage the disease. In the field trials, fungus pressure was managed using BCAs while creating disease in the lentil genotypes compared to untreated control plants.

MATERIALS AND METHODS

Isolation of Fungus: Infected plants were collected from different areas and brought into Lab. Plant samples were disinfected with 70% ethanol and placed on PDA media. The samples were incubated for 20 days at 22±2°C. After three days, the fungus was purified by picking a hypha tip and placed on fresh media plates. Purification of the fungal pathogen was done by using single mycelium transfer and hyphal tip techniques. The fungus culture was identified on the basis of previous reports, morphological and microscopopic characteris like colony color, colony shape, pattern of growth, type of condia, size of spore and overall presentation of spores under microscope (Keogh *et al.*, 1980). After one week, spores were harvested using chilled water, and then the spores were seapared from the debris by passing through 3-4 layers of sterile muslin cloth. Spores of *A. lentis* were counted by adding 10µl inoculum on a hemocytometer under a microscope. The inoculum was adjusted to 10^5 spores/ml by addition of distilled water.

***In-vitro management of A. lentis*:** In-vitro management of *A. lentis* was done by using commercially available fungicides. For this, commercially available chemicals (Tropsi M, Segawin, difenoconazole, Sulphur, thiomyl, dolomite, cymoxinal mancozeb and metalaxyl mancozeb) were diluted to 250mg/ml, 125 mg/ml, 62.5 mg/ml 31.2 mg/ml 15.6 mg/ml 7.8 mg/ml 3.9 mg/ml in distilled water and tested as 50 µl against 50 ul spore suspension in well of ELISA plate by keeping volume upto 200 µl with PDB media. The same was performed for all dilutions; three replications of the experiment were followed for each chemical. For positive control, water was added along with media and spore suspension. Readings of optical density (OD) at the wavelength of 600 nm were recorded; plates were incubated at 25±2ºC. After incubation, readings at different time points were taken by spectrophotometer (Abbas et al., 2013). Biocontrol agents like *Trichoderma harzianum*, *T. viride PsJN* bacteria and non-pathogenic *Aspergillus flavus* were screened on lentils-agar media plates at 25±2ºC as dual culture. *T. Harzianum* significantly inhibited the growth of *A. lentis* on artificial media plates and was further selected to use in management trials.

Sowing of Lentils genotypes: Field trials were conducted in the experimental area of the Department of Plant Pathology. The soil was tested with a hygrometer for moisture analysis. Soil analysis was done at the Institute of Soil and Environmental Sciences and found that soil loam with a ratio of sand, 40% silt, 22% and clay 38%. Twelve advanced lines, including commercially grown cultivars, were collected from Ayub Agricultural Research (Pulses Research Institute), Faisalabad. The germplasm was sown with P-P distance 6 inche and R-R 1.5 feet in 3 replications under RCBD design. Genotypes were also grown in pots in six replications. Fungus A. lentis was isolated from soil samples as well as diseased plant samples prior to field trials. For natural screening of the genotypes were sown in soil containing pathogen as was confirmed during isolation of pathogen from soil. All other agronomic practices and field operations were routinely performed, and the disease index was recorded.

***Inoculation tests on plants*:** Artificial inoculum of A. lentis 10^5spores/ml was given at the basal part of plant near roots by drenching and to the aerial parts through sprayer when the age of plant was 4-6 weeks. The concentration of spores was recorded at 10^8 spores/ml with the help of a Haemocytometer. Mock treatment on control plants was done with distilled water.

***Physiological Parameters*:** Ten days after applying inoculum of A. lentis, BCA like *T. harzianum* and *Aspergillus flavus* in pots, different physiological parameters (shoot length, root length, number of seeds per pod and Pod length) and biomass (Relative water content and cell membrane stability) of the individual plant were recorded and analyzed by using respective formulas. Such as Ali *et al.*, (2011).

***Relative water content*:** Relative water content was measured for detached leaves by calculating from top leaves by following the formula RWC(%)=(FW–DW)/(TW–DW)×100. The fresh weight was calculated after the excision of leaves and for the turgid weight (TW), leaves were soaked in water for 24 h at 4 ºC for rehydration under darkness. Dry weight was taken by placing leaves in the oven for 48 h at 80 ºC.

Cell membrane stability (CMS): CMS was measured based on the phenomenon of electrolyte leakage from the leaf cell by following protocol determined by Ali et al. (2011). In this technique, 0.4g fresh leaves of lentil (0.5 cm diameter leaf discs) were washed with D3 water and placed in test tube and volume was adjusted to 20 ml by D3 water and reading as electrical conductivity (L1) of solution containing leaves were taken. Samples were then autoclaved at 121°C for 20 mint and electrical conductivity (L2) were taken when temperature of the material went down to 25°C. The CMS percentage of sample were recorded by using the following formula:

$$CMS\ (\%) = [(1-(L1/L2)] \times 100$$

RESULTS

Screening of Lentil against the fungal pathogen was done under natural conditions and data were recorded and analyzed further. The results of the screening are given below. Lines 11507 and 11509 show the minimum number of seeds. Line 12505 showed the maximum pod number per plant, while line 12503 showed the least no. of the pod. Line 12503 showed the maximum shoot length, while line M-09501 showed the least height. Line 12503 showed the full root length, while line 12514 showed the least.

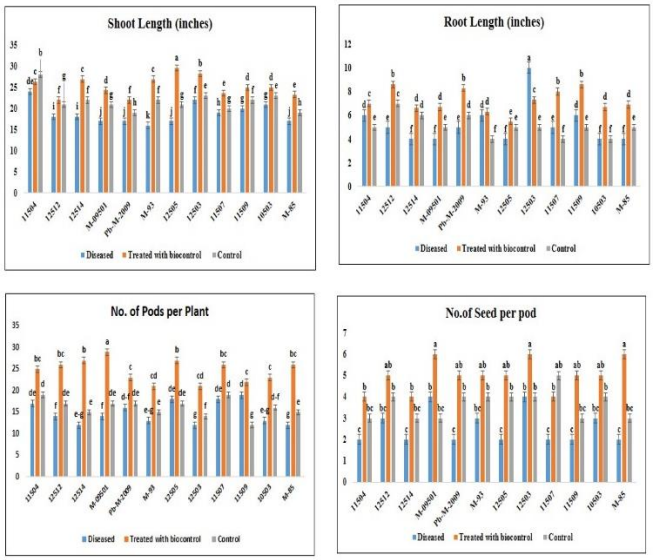
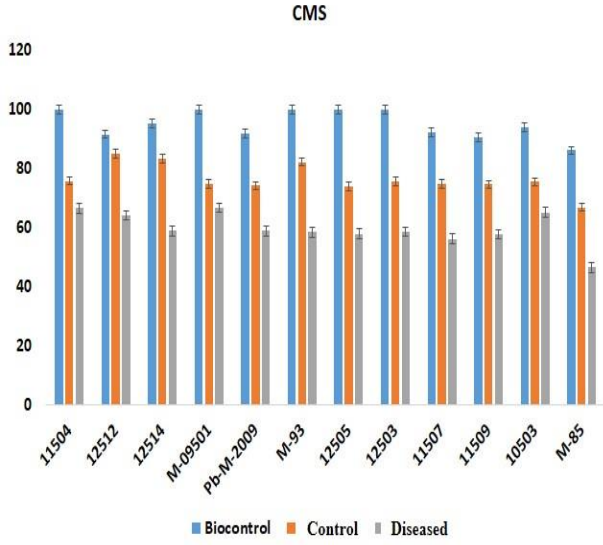


Figure 1. The bar graph shows the response of lines under natural inoculum of the *A. lentis* pathogen shoot length, root length, no. of seeds per pod and No. of pods per plant Graph 1. The bar graph shows the response of lines under artificial inoculum of the *A. lentis* pathogen Graph 3.

***Physiological parameters of Lentil treated with biocontrol*:** All lines show the maximum number of seeds per pod, while lines 11507 and 11509 show the least. Line 12503 shows the maximum length, while line 11509 shows the least. Line 12512 showed the maximum root length while line 11504 showed the least. Line 12505 showed the maximum shoot length while the line 12514 shown the least height.



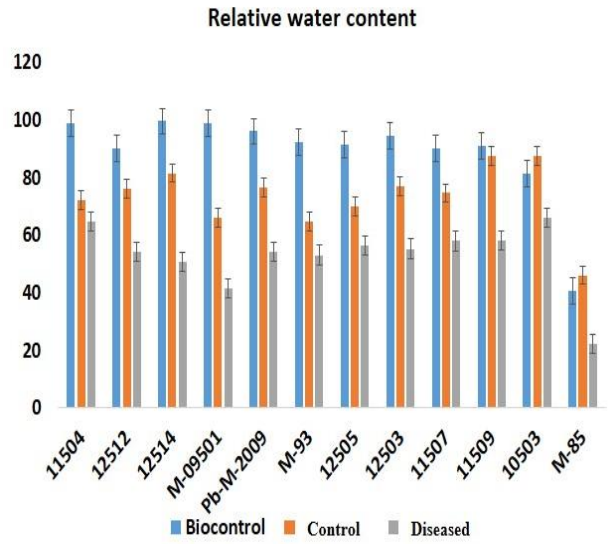


Figure 2. Cell membrane stability and relative water content shows that plants treated with biocontrol had enough resistance towards membrane stability while plants with disease has less resistance.

***Chemical control*:** Results of chemical inhibition percentage at various levels and results are given below. Results showed that all chemicals are best at level 1 because of the high fungicide concentration. At the same time, all fungicides performed well at levels 4 and 5 while dolomite and difenoconazole did not show 50% results.

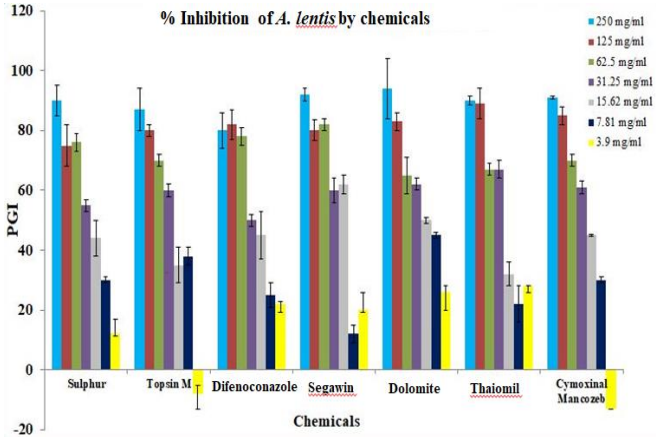


Figure 3. The bar graph shows the response of fungicide levels against the *A. lentis* pathogen.

DISCUSSION

Chaudhry *et al.* (2007) screened 196 lentil's germplasm for resistance against wilt and results shown 100% susceptiblitity amoung the germplasm. Likewise genotypes were screened against Ascochyta blight and similar results were recorded. Lentil screening against Ascochyta blight shown the prevelance of pathogen is high in field. Current study indicates the resistance in lentil germplasm to Ascochyta blight is not infrequent.

Under greenhouse conditions, Ayyub *et al.* (2001) screened out 101 lentil lines against blight, only 5 were resistant, 6 were moderate susceptibility, 13 were susceptible, and 74 were highly susceptible. Similarly, out of 101 lines, 9 lines couldn't show any appearance of infecion symptom under field condition. In contrast, 8 lines found as resistant and 7 as moderately resistants. Remaining were found to be vulnerable to highly vulnerable. Iqbal *et al.* (2005) reported resistant source against AB in the lentil germplasms initiating from national and international research institutes. He recognized 14 lentil lines having resistance against wilt at seedling stage, but not a single line was found as resistant at reproductive stage. Ayyub *et al.* (2003) described highly resistant in lentil germplasm inventing from different source. Our finding about genotype screening were not different from the report mentioned above; furthermore, the effect of different fungicide validates prior report.

Trichoderma species are well recognized as soil-managing fungi against various soil-inhibiting fungal pathogens. (Schuster and Schmoll, 2010). *Trichoderma* is commonly used as a biocontrol agent due to its antimicrobial properties, including parasitism and competition. (Sivakumar, 2001). Trichoderma can produce various compounds like enzymes, proteins and antibiotics to manage pathogenic fungi. (Al-Taweil *et al.*, 2009; Vinale *et al.*, 2008). Aroosa *et al.* (2012) tested 14 chemicals against the Fusarium wilt of tomato and found a 20-80% growth reduction of *Fol* under in-vitro conditions. The strobilurin group's Picoxystrobin fungicide helps inhibit mitochondrial respiration by blocking electron transfer in the bc1 complex of conveying current for mitochondrial electrons.

***Conclusion*:** The present study was conducted to find an effective biocontrol agent against fungal pathogen of Lentil. Results indicate that these biocontrol agents not only help to deal with disease but also increase vegetative growth and yield of the crop. Results also give effective fungicidal dose with minimum chemical concentration, making it eco-friendly.

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